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Determination of Genotypic Diversity of *Mycobacterium avium* Subspecies from Human and Animal Origins by Mycobacterial Interspersed Repetitive-Unit–Variable-Number Tandem-Repeat and IS*1311* Restriction Fragment Length Polymorphism Typing Methods▽†

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Members of the *Mycobacterium avium* complex (MAC) are ubiquitous bacteria that can be found in water, food, and other environmental samples and are considered opportunistic pathogens for numerous animal species, mainly birds and pigs, as well as for humans. We have recently demonstrated the usefulness of a PCR-based mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) typing for the molecular characterization of *M. avium* subsp. *paratuberculosis* and *M. avium* strains exclusively isolated from AIDS patients. In the present study we extended our analysis, based on eight MIRU-VNTR markers, to a strain collection comprehensively comprising the other *M. avium* subspecies, including *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, and *M. avium* subsp. *silvaticum*, isolated from numerous animal species, HIV-positive and HIV-negative humans, and environmental sources. All strains were fully typeable, with the discriminatory index being 0.885, which is almost equal to that obtained by IS*1311* restriction fragment length polymorphism (RFLP) typing as a reference. In contrast to IS*1311* RFLP typing, MIRU-VNTR typing was able to further discriminate *M. avium* subsp. *avium* strains. MIRU-VNTR alleles strongly associated with or specific for *M. avium* subspecies were detected in several markers. Moreover, the MIRU-VNTR typing-based results were consistent with a scenario of the independent evolution of *M. avium* subsp. *avium*/*M. avium* subsp. *silvaticum* and *M. avium* subsp. *paratuberculosis* from *M. avium* subsp. *hominissuis*, previously proposed on the basis of multilocus sequence analysis. MIRU-VNTR typing therefore appears to be a convenient typing method capable of distinguishing the three main subspecies and strains of the complex and providing new epidemiological knowledge on MAC.

The most frequent agents of nontuberculous mycobacterioses belong to the *Mycobacterium avium* complex (MAC); in particular, *M. avium* subsp. *hominissuis* is a frequent agent of human mycobacterioses (12, 25). Members of this subspecies are also frequent infectious agents for pigs, leading to significant economic losses in pig farming, albeit that subspecies produces very low rates of morbidity in this animal species (23, 24). Two other MAC members, *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis*, are the causative agents of two other important, often fatal (2) animal pathologies, avian tuberculosis (40) and ruminant paratuberculosis (Johne's disease) (6), respectively. Like other opportunistic agents, *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis* are also capable of infecting a wide range of animal species, including cattle, deer, wild boars, goats, and horses (40). In contrast, *M. avium* subsp. *silvaticum* is taxonomically very close to *M. avium* subsp. *avium* but almost exclusively infects wood pigeons (41).

In the particular case of *M. avium* subsp. *hominissuis*, strains with similar or identical genotypes are usually found in common between pigs and human patients (26), which does not permit the potential zoonotic risk of this subspecies to be discarded. Moreover, these mycobacteria can be found in environmental sources such as water, biofilms, soil, aerosols, and phagocytic protozoa and amoebae (11), all of which can act as common sources of infection for animals and humans.

For epidemiological investigations of MAC, the current reference molecular typing technique is restriction fragment length polymorphism analysis (RFLP) based on the IS*1245* (47) and IS*1311* (19, 20) insertion sequences. Whereas IS*1311*

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RFLP usually generates clear hybridization patterns, IS*1245* RFLP yields complex multiband patterns which are difficult to compare among different experiments and laboratories, mainly because of the heterogeneity in the intensities of the hybridization bands (19, 20, 42). Recently, an even simpler PCR-based molecular typing method, multilocus variable-number tandem-repeat analysis (MLVA), which is based on mycobacterial repetitive elements called mycobacterial interspersed repetitive-unit–variable-number tandem repeats (MIRU-VNTRs) (14, 34, 36, 37), has been described for *M. avium* subsp. *paratuberculosis* (38). This method presented better results for the differentiation of strains of this subspecies than those obtained by the standard IS*900* RFLP method (38) and showed a promisingly good discrimination index (DI) with a panel of *M. avium* strains isolated from human AIDS patients (38).

In the study described here, we extended that initial study by applying MIRU-VNTR typing to a large strain panel set comprising *M. avium* subsp. *hominissuis*, *M. avium* subsp. *avium*, and *M. avium* subsp. *silvaticum* strains isolated from diverse animal and human sources. Our aim was to further analyze the power of MIRU-VNTR typing to discriminate isolates within these subspecies and to identify possible specific signatures within the complex for better characterization and detection of interspecies transmission patterns.

MATERIALS AND METHODS

Strain collection and species identification. A collection of 117 strains was assembled for this study: 62 isolated from domesticated pigs, 9 from wild birds, 4 from wood pigeons, 8 from poultry, 14 from cattle, 5 from HIV-positive (HIV^+) patients, 9 from HIV-negative (HIV^-) patients, 2 from wild boars, 1 from a kangaroo, 1 from a goat, 1 from a cat, and 1 from a soil sample (see Table S1 in the supplemental material for details). The human isolates belonged to the Pasteur Institute (Paris, France) collection (31). The animal isolates belonged to the Animal Mycobacterioses National Reference Laboratory (AFSSA; Maisons-Alfort, France). All strains except the strains isolated from wood pigeons were grown until they reached stationary phase in 10 ml Middlebrook 7H9 broth without mycobactin. For the strains isolated from wood pigeons, considered to be *M. avium* subsp. *silvaticum*, mycobactin J was added. Strain identification was performed by biochemical and culture phenotypic characterization, which does not distinguish *M. avium* from *M. intracellulare* strains (8). In order to retain only taxonomically recognized *M. avium* strains and discard any *M. intracellulare* strains from our collection, a PCR targeting IS*1245*, which is present in *M. avium* genomes but not in *M. intracellulare* genomes (15, 19), was performed as described previously (19). Likewise, *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, and *M. avium* subsp. *hominissuis* were distinguished by testing for the presence of IS*901* (22), which is present in the *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* genomes but not in the *M. avium* subsp. *hominissuis* genome (10, 21, 30). One hundred eighty-three *M. avium* subsp. *paratuberculosis* isolates previously studied by Thibault et al. (38) were additionally considered for analysis of the MIRU-VNTR typing results.

DNA extraction. DNA was extracted as described previously (1), with the following modifications. The cell pellets from the centrifugation of 10 ml of concentrated culture were washed in 1 ml of 1× TE buffer (10 mM Tris, pH 7.6, 1 mM EDTA). After a second centrifugation, the cells were resuspended in 400 μl of 1× TE buffer and lysed by addition of 50 μl of 10 mg/ml lysozyme and 12 h of incubation at 37°C with agitation at 650 rpm, followed by addition of 70 μl of 10% SDS and 5 μl of 10 mg/ml proteinase K and 10 min of incubation at 65°C with agitation at 650 rpm. Subsequently, 100 μl of 5 M NaCl and 100 μl of cetyltrimethylammonium bromide-NaCl (10%/0.7 M) preheated at 65°C were added, followed by addition of 750 μl of isoamyl-chloroform (24/1). The aqueous phase was recovered by using Phase Lock Gels 5′-3′ (Eppendorf). DNA was precipitated with 450 μl of isopropanol and then centrifuged at 15,000 × *g* for 15 min at 18°C and washed with 200 μl of cold 70% ethanol. After evaporation of the ethanol at 37°C, the DNA was resuspended in 1× TE buffer and standardized at a concentration of 50 ng/μl by use of a Biophotometer apparatus (Eppendorf).

MIRU-VNTR typing. MIRU-VTNR typing was performed by using the eight loci identified as polymorphic for *M. avium* subsp. *paratuberculosis* K10 and called MIRU or VNTR 292, X3, 25, 47, 3, 7, 10, and 32, as described previously (38). Repeat numbers (alleles) were determined according to the amplified fragment sizes by the use of Gel Doc 2000 (Bio-Rad) and Quantity One 4.2.1 (Bio-Rad) software for fragment size calculation and according to the results obtained with a previously described allele-calling table (38). New profiles were assigned INRA, Nouzilly, MIRU-VNTR (INMV) codes (38).

IS*1311* RFLP typing. IS*1311* RFLP typing was performed as described previously (19, 20). Briefly, an IS*1311* probe was obtained by PCR of the *M. avium* 104 reference strain (GenBank accession number NC_008595) genome, purified with QIAquick (Qiagen), and labeled by using an enhanced chemiluminescence direct labeling and detection system (Amersham, General Electric Healthcare). Genomic DNA (1 to 2 μg) was digested with the PvuII enzyme (Promega). The migrations of the restriction fragments, resolved on 0.8% agarose gels, were standardized by using a 1-kb Plus Ladder (Invitrogen) as a molecular size marker (wt/vol). The restriction fragments were blotted onto a Nytran membrane (Schleicher-Schuel) by vacuum transfer, and hybridized DNA fragments were detected according to the manufacturer's recommendations.

Strain comparison. The IS*1311* RFLP and MIRU-VNTR patterns were analyzed by using Bionumerics software (version 5.0; Applied Maths, St-Martens-Latem, Belgium). Dendrograms for strain genotype comparisons were obtained by using individual or combined IS*1311* RFLP and MIRU-VNTR results and the unweighted-pair group method with arithmetic averages (UPGMA) clustering method. Categorical and Dice coefficients were used for the MIRU-VTNR and IS*1311* RFLP (with 3% optimization, 3% size tolerance, and visual inspection) methods, respectively. As described previously (39), the MIRU-VNTR minimum-spanning tree was calculated by using the maximum number of single-locus variants (SLVs) as a priority parameter.

Calculation of discriminatory power. The DIs for each typing methods and their combination were calculated by use of the formula described by Hunter and Gaston (16). MIRU-VNTR allelic diversity was calculated by using Nei's index (27).

RESULTS

MIRU-VNTR typing. Twenty-three MIRU-VNTR patterns, called INMVs (38), were identified for the 81 *M. avium* subsp. *hominissuis* isolates and 8 were identified for the 32 *M. avium* subsp. *avium* isolates, while a single pattern was observed for the 4 *M. avium*. subsp. *silvaticum* isolates (Fig. 1; see Table S1 in the supplemental material). None of the patterns deduced for *M. avium* subsp. *avium* matched those of *M. avium* subsp. *hominissuis* or *M. avium* subsp. *silvaticum*. Moreover, none of these MIRU-VNTR profiles matched those previously identified in *M. avium* subsp. *paratuberculosis* (38). In contrast, two of the *M. avium* subsp. *avium* strain patterns and seven of the *M. avium* subsp. *hominissuis* strain patterns were identical to the MIRU-VNTR genotypes found among other, previously analyzed *M. avium* strains isolated from AIDS patients (38) (see Table S1 in the supplemental material). Among these, INMV patterns 51 and 43 consistently represented the majority of the isolates in both studies (39% and 19% of the *M. avium* subsp. *hominissuis* isolates analyzed in the present study, respectively, and 26% and 29% of the *M. avium* isolates analyzed in the previous study, respectively). In addition, 23 genotypes were newly identified (see Table S2 in the supplemental material).

The allelic diversities were higher for virtually all MIRU-VNTR markers in *M. avium* subsp. *hominissuis* than in *M. avium* subsp. *avium*, and only two markers were monomorphic in the former species, whereas five markers were monomorphic in the latter species (Table 1), in keeping with the 3-fold higher number of *M. avium* subsp. *hominissuis* genotypes. In terms of the discriminatory hierarchy, locus X3 displayed the highest allelic diversity for both *M. avium* subsp. *hominissuis* and *M. avium* subsp. *avium*. Interestingly, in the latter case, this

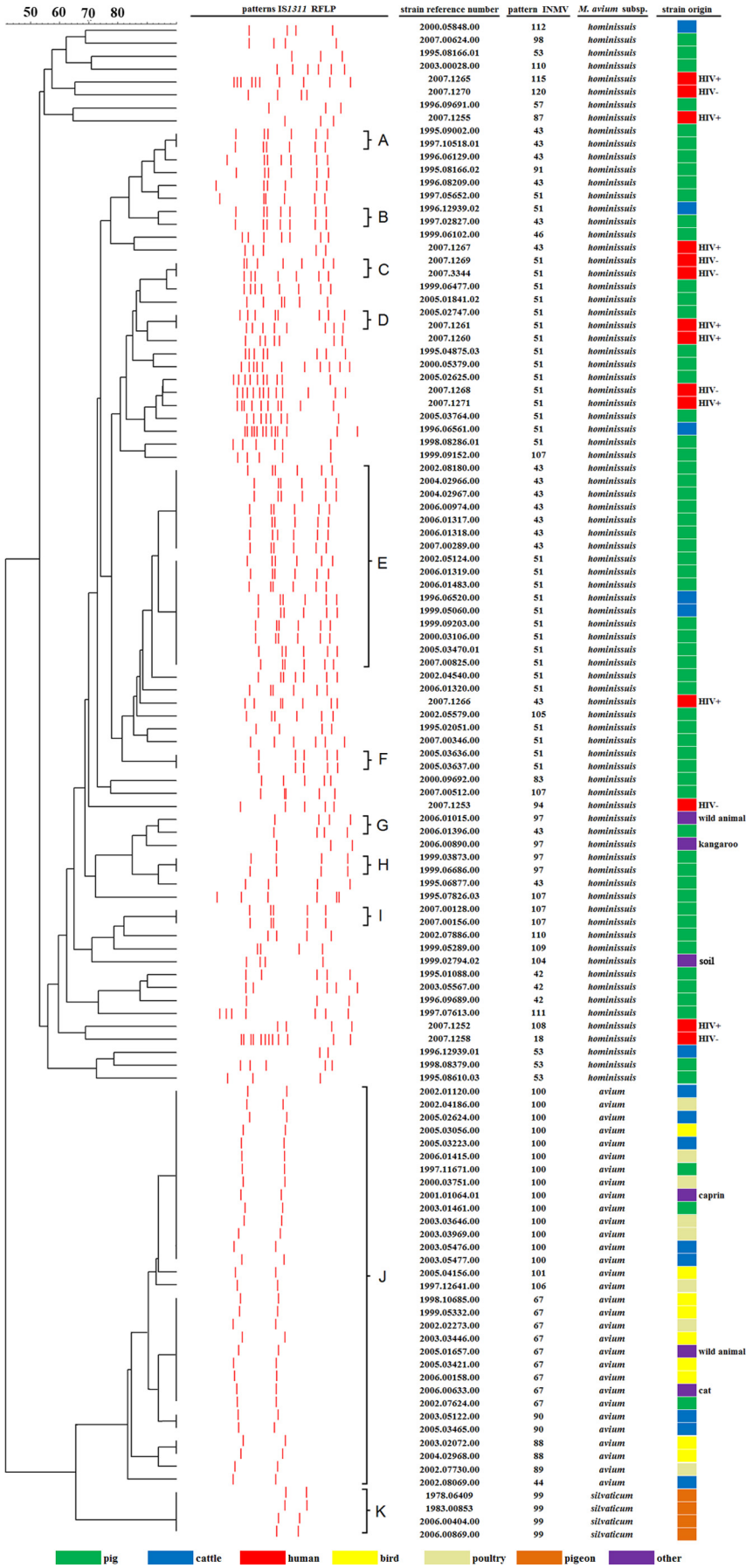
50 60 70 80
patterns IS1311 RFLP
strain reference number
pattern INMV
M. avium subsp.
strain origin
A
B
C
D
E
F
G
H
I
J
K
pig
cattle
human
bird
poultry
pigeon
other

TABLE 1. MIRU-VNTR allelic distribution among *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *hominissuis*, and *M. avium* subsp. *paratuberculosis* isolates

Strain panel	Locus	No. of isolates with the following MIRU-VNTR copy no.:											Allelic diversity (h)[a]
		0	1	2	3	4	5	6	7	8	9	10	
All subspecies[b]	X3		3	25	16	55	17		1				0.69
	25		35	69	12	1							0.55
	32						4		35	75	3		0.49
	47			73	44								0.46
	10		8	103	2		4						0.21
	292	6	1	108	2								0.14
	3		117										0
	7		117										0
M. avium subsp. *avium*	X3		3	2	10	15	1						0.64
	25		31										0
	32								30	1			0.03
	47				31								0
	10		3	27	1								0.21
	292			31									0
	3		31										0
	7		31										0
M. avium subsp. *silvaticum*	X3					4							0
	25		4										0
	32								4				0
	47				4								0
	10		4										0
	292			4									0
	3		4										0
	7		4										0
M. avium subsp. *hominissuis*	X3			23	6	36	16		1				0.68
	25			69	12	1							0.26
	32						4		1	74	3		0.17
	47			72	10								0.20
	10		1	76	1		4						0.13
	292	6	1	73	2								0.19
	3		82										0
	7		82										0
M. avium subsp. *paratuberculosis*[c]	X3	3	179	1									0.04
	25				176	1	6			67	8		0.07
	32									177	5	1	0.59
	47			5	178								0.05
	10		19	164									0.18
	292			6	108	69							0.51
	3	1		182									0.005
	7		12	164	3	2	2						0.19

[a] Calculated by using Nei's index (27). h, genetic diversity.
[b] With the exception of *M. avium* subsp. *paratuberculosis*, which was not integrated into the current study.
[c] Data are from Thibault et al. (38).

locus actually provided most of the discriminatory power *per se*, as the only two other informative markers (markers 10 and 32) displayed a distribution highly skewed toward one strongly overrepresented allele among two or three alleles, at most. At the other extremity, markers 3 and 7 were completely monomorphic among all the *M. avium* strains in our panel.

The allelic signatures strongly or strictly associated with the subspecies. Indeed, several loci were found to be monomorphic in certain taxons. For instance, allele 1 of marker 25 was exclusively restricted to and shared by all *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* strains, while all *M. avium* subsp. *hominissuis* and *M. avium* subsp. *paratuberculosis*

FIG. 1. Dendrogram based on combined IS*1311* RFLP typing and MIRU-VNTR typing of *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, and *M. avium* subsp. *silvaticum*. One hundred seventeen strains were isolated from pigs (green), cattle (blue), humans (red), poultry (white), birds (yellow), wood pigeons (brown), and other sources, such as wild animals, a kangaroo, a cat, a goat, and a soil sample (purple). The color codes of the sample origins are also given at the bottom. MIRU-VNTR typing was done by using eight loci, and the corresponding INMV patterns are indicated. Clusters of IS*1311* RFLP patterns considered identical (within the size tolerance limits) are coded from A to K.

TABLE 2. Discrimination index (DI) of IS*1311* RFLP, MIRU-VNTR used alone, and RFLP and MIRU-VNTR in combination among *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, and *M. avium* subsp. *silvaticum* isolates

Strain panel	Typing method	No. of different patterns	No. of clusters	No. of clustered isolates	No. of unique isolates	No. of isolates in each cluster	DI[a]
All subspecies	RFLP	61	11	67	50	2–31	0.912
	MIRU-VNTR	32	12	97	20	2–32	0.885
	RFLP + MIRU-VNTR	71	13	59	58	2–14	0.971
	RFLP	1	0	0	31		0
M. avium subsp. *avium*	MIRU-VNTR	8	4	27	4	2–14	0.723
	RFLP + MIRU-VNTR	8	4	27	4	2–14	0.723
	RFLP	59	9	32	50	2–16	0.961
M. avium subsp. *hominissuis*	MIRU-VNTR	23	7	66	16	2–32	0.807
	RFLP + MIRU-VNTR	62	8	28	54	2–9	0.981
	RFLP	1	0	4	0	4	0
M. avium subsp. *silvaticum*	MIRU-VNTR	1	0	4	0	4	0
	RFLP + MIRU-VNTR	1	0	4	0	4	0

[a] Calculated as described by Hunter and Gaston (16).

strains contained two or more repeats in this locus (Table 1; see Table S1 in the supplementary material). Likewise, only minimal overlap was detected among the allelic distributions of locus 32. All of the *M. avium* subsp. *silvaticum* strains and all but one of the *M. avium* subsp. *avium* strains shared allele 7 in this marker, while all but one *M. avium* subsp. *hominissuis* strain and all but one *M. avium* subsp. *paratuberculosis* strain displayed eight repeats or more. Alleles 3 and 4 of locus 292 were exclusively restricted to and shared by most *M. avium* subsp. *paratuberculosis* strains.

MIRU-VNTR typing versus IS*1311* RFLP typing. As was observed previously (19), two main groups could easily be distinguished by IS*1311* RFLP (Fig. 1): one group comprising the 35 *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* strains of our set presenting simple double-band patterns and another group comprising the 82 *M. avium* subsp. *hominissuis* strains of our collection with multiband patterns. In addition, the 31 *M. avium* subsp. *avium* isolates formed a single large cluster (named cluster J) with identical double-band patterns, which was distinct from the double-band pattern (cluster K) shared by the 4 *M. avium* subsp. *silvaticum* isolates. In contrast, within the group of *M. avium* subsp. *hominissuis* strains, only one large cluster (cluster E) of 16 isolates was observed, whereas clusters of no more than 2 strains (clusters A to D and F to I) were otherwise found.

The four strains of *M. avium* subsp. *silvaticum* were identically clustered by IS*1311* RFLP and MIRU-VNTR typing into patterns K and 99, respectively (Fig. 1). Five other small IS*1311* RFLP clusters (clusters A, C, D, F, H, and I), including *M. avium* subsp. *hominissuis* strains, were concordantly matched by MIRU-VNTR clusters.

Only two small *M. avium* subsp. *hominissuis* IS*1311* RFLP clusters (clusters B and G) were subdivided by MIRU-VNTR loci, and in each case this was only by an SLV. Likewise, large IS*1311* RFLP cluster E was subdivided into two MIRU-VNTR types, INMV patterns 43 and 51, that differed only by an SLV. In contrast, cluster J, which included the 31 *M. avium* subsp. *avium* isolates, was divided into eight MIRU-VNTR types (INMV patterns 44, 67, 88, 89, 90, 100, 101, and 106) that differed by one to three loci.

Conversely, the main MIRU-VNTR clusters, corresponding to INMV patterns 51 and 43 patterns, were divided by IS*1311* RFLP into 21 and 9 different profiles, respectively. Some other smaller clusters were likewise subdivided: INMV patterns 53 and 107 into four profiles, INMV 97 and 42 into three profiles, and 110 into two profiles (see Table S1 in the supplemental material).

Discrimination indexes were calculated for each typing method and for the two methods in combination for the strain collection as a whole and for each subspecies individually (Table 2). Each method separately provided very similar discrimination indexes (Table 2) for the total collection (0.885 for MIRU-VNTR versus 0.912 for IS*1311* RFLP). The slightly better resolution power of IS*1311* RFLP was explained by the better performance of IS*1311* RFLP with *M. avium* subsp. *hominissuis* (0.961 versus 0.807 for MIRU-VNTR), which was better represented in this strain collection. This difference was therefore only partly compensated for by the clearly superior discriminatory power of MIRU-VNTR typing among *M. avium* subsp. *avium* strains (0.723 versus 0 for IS*1311* RFLP). As a result of the ability of each method to subdivide some of the clusters defined by the other method, use of the combination of IS*1311* RFLP and MIRU-VNTR gave maximal resolution power for the overall strain collection. However, this additive effect was smaller when only *M. avium* subsp. *hominissuis* was considered, as a result of the less frequent cross-subdivision of clusters obtained by the two methods for this taxon (see above).

Phylogeny and host preference analysis. As it provides a high resolution power and is based on multiple independent markers, MIRU-VNTR typing is especially suitable for analysis of clonal complexes and phylogenetic groupings in closely related subspecies when the minimum-spanning-tree approach is used (Fig. 2). Two consistent MIRU-VNTR-based complexes were clearly identified and distinguished (complexes B and C in Fig. 2) among the *M. avium* isolates, completely separating IS*901*-negative strains considered to be *M. avium* subsp. *hominissuis* (dark gray) and IS*901*-positive strains considered to be *M. avium* subsp. *avium* or *M. avium* subsp. *silvaticum* (light gray). The *M. avium* subsp. *hominissuis* complex

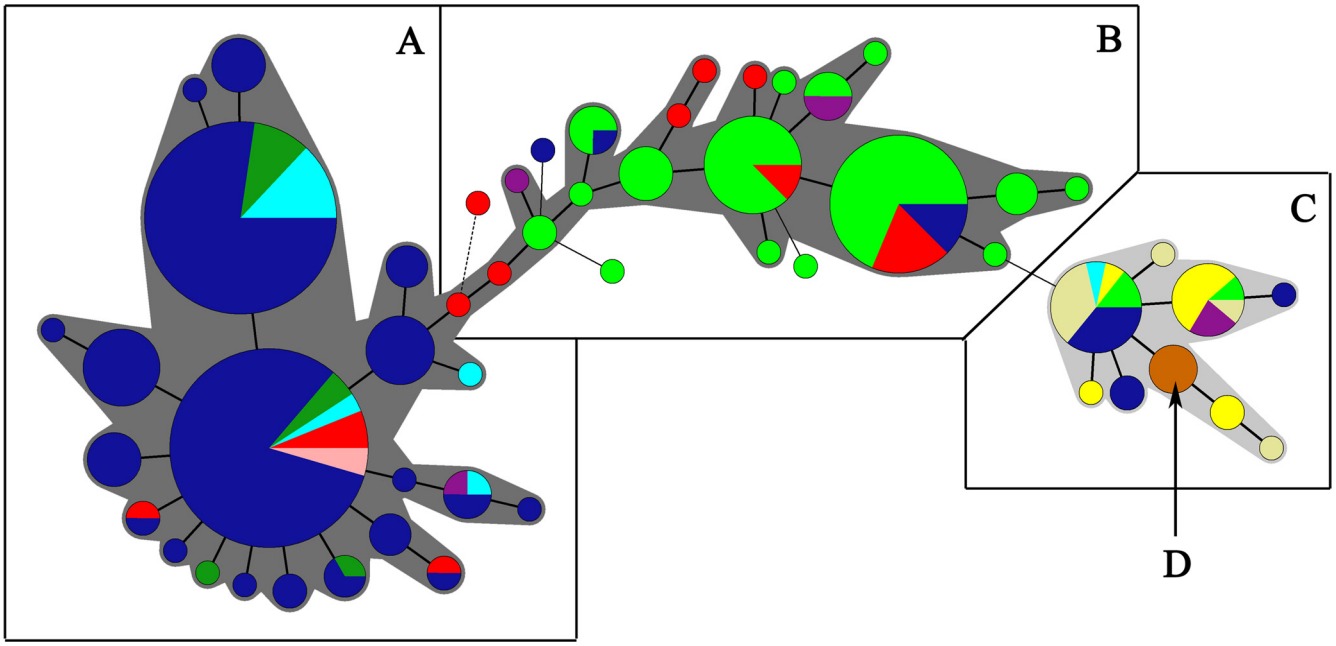

FIG. 2. Minimum spanning tree based on MIRU-VNTR typing results for *M. avium* subsp. *paratuberculosis* (A), *M. avium* subsp. *hominissuis* (B), *M. avium* subsp. *avium* (C), and *M. avium* subsp. *silvaticum* (D) isolates. Circles correspond to the different patterns identified by this method among 183 isolates from the study of Thibault et al. (38) (A) and 117 isolates from this study (B, C, and D); circle sizes are proportional to the numbers of isolates sharing an identical pattern. Strains were isolated from pigs (light green), cattle (dark blue), humans (red), poultry (white), birds (yellow), wood pigeons (brown), goats (light blue), sheep (pink), deer (dark green), and other sources (wild animals, a kangaroo, a soil sample, a cat, and a rabbit) (purple). Gray zones include MIRU-VNTR patterns with SLVs, corresponding to *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *hominissuis* (dark gray) and *M. avium* subsp. *avium*/*M. avium* subsp. *silvaticum* (light gray). Linkages by SLV, double-locus, and triple-locus variations are indicated with thick lines, thin lines, and dotted lines, respectively.

was mostly composed of pig strains but also comprised the totality of human strains. The other complex, that of *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum*, included all the bird isolates. Within each complex, no correlation between genotype groupings and host origin or clinical status was observed among the human isolates.

Furthermore, these two *M. avium* complexes were also separated from the complex that comprised the MIRU-VNTR genotypes of the 183 *M. avium* subsp. *paratuberculosis* isolates (Fig. 2, complex A). This *M. avium* subsp. *paratuberculosis* branch mainly includes cattle isolates, as a result of their abundance in the corresponding strain collection (38).

DISCUSSION

The subspecies division of *M. avium* has been studied by molecular typing techniques, such as by the identification and analysis of subspecies-associated insertion elements (45) and MLSA based on gene sequence variation (43, 44). In the present study, we used a large set of *M. avium* isolates from different sources, including humans, pigs, and cattle, as well as rarely studied isolates from birds, poultry, and wood pigeons, to evaluate the relevance of the MIRU-VNTR markers, initially identified in *M. avium* subsp. *paratuberculosis* (38), in order to (i) distinguish strains of the different subspecies, (ii) examine the genetic consistency of taxonomic separations, and (iii) analyze the correlation between genotype and host origin.

Interestingly, all the strains in the *M. avium* panel used in the present study were fully typeable by the use of these eight MIRU-VNTR markers when the same set of primers designed on the basis of the *M. avium* subsp. *paratuberculosis* genomic sequence was used. This observation points to both the important genetic conservation among the *M. avium* subspecies, in line with previous evidence (43), and the general applicability of these MIRU-VNTR markers for the whole *M. avium* species.

To evaluate the resolution power of the MIRU-VNTR markers, IS*1311* RFLP instead of IS*1245* RFLP was used as a reference method, because the former RFLP technique provides more easily interpretable patterns (especially for *M. avium* subsp. *hominissuis*) and has discriminatory power almost equal to that of the latter one (19, 20). MIRU-VNTR and IS*1311* RFLP typing overall provided very similar discrimination indexes for the total collection, but the maximal resolution power for the total strain collection was obtained by using the combination of both methods. Whereas IS*1311* RFLP typing offered the better resolution of *M. avium* subsp. *hominissuis* isolates, only MIRU-VNTR typing was capable of discriminating *M. avium* subsp. *avium* strains. In the latter case, the corresponding large IS*1311* RFLP cluster (cluster J) was subdivided by one to three MIRU-VNTR markers, while three other IS*1311* RFLP clusters in the rest of the collection were subdivided by SLVs. The other six RFLP clusters were fully matched by MIRU-VNTR clusters. Although it was not possible to test the clonal stability of the MIRU-VNTR markers with this set of *M. avium* strains, the same markers were found to be clonally stable among different *M. avium* subsp. *paratu-*

berculosis vaccine batches (38). This suggests that most of the subdivisions of IS*1311* RFLP clusters by MIRU-VNTR markers (even by SLVs) are probably epidemiologically meaningful; i.e., they reflect infection by different strains. The same conclusion has been reached for the interpretation of MIRU-VNTR genotypic differences between *M. tuberculosis* isolates, based on the analysis of large sets of epidemiologically well defined strains (33, 35, 46).

Most of the discriminatory power of typing by MIRU-VNTR analysis was clearly concentrated in a few more variable markers, especially at the infrataxon level. Interestingly, however, allelic signatures strictly or strongly associated with subspecies were identified for several MIRU-VNTR markers, including some monomorphic loci within taxons (e.g., alleles 1 and ≥2 of marker 25 were restricted to *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* strains and *M. avium* subsp. *hominissuis* and *M. avium* subsp. *paratuberculosis* strains, respectively; alleles 3 and 4 of locus 292 were restricted to most *M. avium* subsp. *paratuberculosis* strains) (Table 2). As a result of this minimal overlap among the allelic distributions between the different subspecies, MIRU-VNTR-based genotype complexes were found to perfectly correlate with the separation of IS*901*-negative strains considered to be *M. avium* subsp. *hominissuis* and IS*901*-positive strains considered to be *M. avium* subsp. *avium* or *M. avium* subsp. *silvaticum* (Fig. 2). In turn, these two complexes were perfectly separated from the complex grouping 183 *M. avium* subsp. *paratuberculosis* isolates typed with the same MIRU-VNTR markers (38).

MLSA of 56 strains indicated more genetic variability and a more nonclonal population structure in *M. avium* subsp. *hominissuis* than in *M. avium* subsp. *avium*/*M. avium* subsp. *silvaticum* and *M. avium* subsp. *paratuberculosis*. The results suggested that *M. avium* subsp. *hominissuis* represents a heterogeneous group of organisms from which the other subspecies have evolved as two independent pathogenic clones (43). The results of MIRU-VNTR-based analysis of our larger collection of 300 isolates (including results published previously [38]) (Fig. 2) are fully consistent with this scenario of independent evolution from a *M. avium* subsp. *hominissuis*-like pool. Within our sampling limits, more variability was also seen among *M. avium* subsp. *hominissuis* isolates, as reflected by a higher number of independently connected genotypes across the minimum-spanning tree. This contrasts with the common connection of many peripheral, minor genotypes to two central, major genotypes for *M. avium* subsp. *paratuberculosis*, indicative of some variants arising from only two major clones (corresponding to INMV patterns 1 and 2 [38]). Thus, analysis of a small set of MIRU-VNTR loci, particularly those that are more phylogenetically informative (i.e., with subspecies-specific allelic distributions; see above), is sufficient for obtaining a taxonomic classification equivalent to that obtained by MLSA or other molecular typing methods (19, 20, 43).

Concerning the relevance of distinguishing *M. avium* subsp. *silvaticum* from *M. avium* subsp. *avium* strains (45), we note that both IS*1311* RFLP and MIRU-VNTR typing distinguished the corresponding isolates in this panel. However, the variations were very limited, as is the case with MLSA (43). Variations consisted of band positions in a common two-band RFLP pattern and MIRU-VNTR SLVs between the respective strain clusters. A clear limitation is that our strain panel and that of Turenne et al. (43) included only four and three strains of *M. avium* subsp. *silvaticum*, respectively, revealing the small number of strains described in the literature. Whether *M. avium* subsp. *silvaticum* can be monophyletically distinguished from *M. avium* subsp. *avium* will require analysis of a larger and more representative set of strains.

As for MLSA (43), the *M. avium* subsp. *hominissuis* MIRU-VNTR-based complex included mostly pig and human isolates, but no bird isolates. This is consistent with the notion that *M. avium* subsp. *hominissuis* is an environmental bacterium that specifically causes opportunistic infections in some mammals, such as swine, and immunocompromised or immunocompetent people with some predisposing factors (3, 7, 13, 29). Therefore, the finding of identical genotypes between pig and human isolates, also observed by IS*1245* RFLP typing (38), may indicate common sources of infection but does not exclude a potential zoonotic risk of this subspecies. Similarly, the *M. avium* subsp. *avium*/*M. avium* subsp. *silvaticum* MIRU-VNTR-based complex included all the bird isolates but also isolates from other animal species, some of which had the same genotypes as the avian strains. This could also be explained by the ubiquitous environmental distribution of these bird pathogens and/or by host spillover effects (43).

Before molecular typing was developed, the different *M. avium* types within the MAC were defined by serotyping. Thirty-one distinct MAC serovars were established by the presence of surface-located glycopeptidolipids (GPLs) harboring various glycosylation patterns (5). More precisely, serotypes 1 to 6, 8 to 11, 21, and 28 were assigned to *M. avium*, either to *M. avium* subsp. *avium* or *M. avium* subsp. *hominissuis*, while serotypes 7, 12 to 20, and 25 were assigned to *M. intracellulare* (10, 18, 25, 48). Many previous reports describing inter-animal species transmission patterns based on such serotyping data have been published, and environmental distribution studies were done on the basis of such serotyping data. Although it is difficult to compare the results obtained by this technique due to its lack of standardization (9), it would be interesting to analyze the correlation between MIRU-VNTR types and serotypes, in order to capitalize on previous knowledge related to strain, virulence, host preference, and zoonotic risk.

In conclusion, we demonstrated here that MIRU-VNTR markers can be used for the subspecies and infra- and intrasubspecies differentiation of strains of any subspecies of MAC. The technical ease of performance of PCR and the numerical genotype format make this method a very useful additional tool that can be applied as a first-line analysis technique or as a complement to MLSA or classical RFLP methods. In addition to our first report, other reports have described the use of MIRU-VNTR markers for the genotyping of MAC strains (4, 17, 28, 32). In particular, Inagaki et al. described the use of some additional VNTR loci for the typing of a panel of *M. avium* subsp. *hominissuis* strains, but these were only strains of human origin (17). Three of our eight markers, namely, MIRU-VNTR 292, 10, and X3, were also used in one or more of those studies. To facilitate the use of these markers and future comparisons between studies, we recommend that the same primers and allele calling system used in our studies be used (see Table S3 in the supplemental material). As for *Mycobacterium tuberculosis* (35), a consensus-based MIRU-VNTR typing method could thus become the new standard for

refined molecular epidemiological screening, to analyze the origins and transmission patterns of *M. avium* mycobacterioses in human and animal outbreaks.

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